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ENHANCED BIOHYDROGEN PRODUCTION BY *Enterobacter cloacae* USING ENZYMATIC HYDROLYSATE FROM CASHEW APPLE BAGASSE: INFLUENCE OF THE SUPPLEMENTATION WITH NITROGEN SOURCES

Gabriel F. Simão[1*], Carla L. B. Reis[1], Larissa X. Nascimento[1], Estefânia S. Moreira[2] & Maria V. P. Rocha[1*]

[1] *Universidade Federal do Ceará, Departamento de Engenharia Química, Fortaleza, Brasil.*
[2] *Universidade da Integração Internacional da Lusofonia Afro-Brasileira, Instituto de Engenharias e Desenvolvimento Sustentável, Redenção, Brasil.*

** Corresponding author's email address: gabrielsimao9@gmail.com; valderez.rocha@ufc.br*

ABSTRACT

Dark fermentation from cashew apple bagasse (CAB) hydrolysate can be exploited as a sustainable alternative source for hydrogen production due to its high carbohydrate content, contributing to decarbonization. In this work, the influence of supplementation of enzymatic hydrolysate of cashew apple bagasse (CAB-EH) with nitrogen source on hydrogen production was evaluated. The glucose concentration of the CAB- EH was adjusted for 20 ± 4 g/L. The cumulative hydrogen obtained in the bioprocesses using CAB-EH without supplementation was 54 mmolH_2/$L_{hydrolysate}$ with 24 h of bioprocess, and the production increased at supplement CAB- EH with 3 g/L peptone (58.9 mmolH_2/L hydrolysate). However, the production decreased drastically using CAB- EH supplemented with 3 g/L urea, achieving only 2 mmolH_2/$L_{hydrolysate}$. The main soluble metabolites were acetic and lactic acids, reaching values of 6.0 ± 0.5 g/L and 1.4 ± 0.2 g/L in the assay using CAB-HE supplemented with peptone. Therefore, the use of CAB was considered a promising strategy in the production of bio-H_2 by dark fermentation and the nitrogen source used influences the metabolic pathway of production of this biofuel by *Enterobacter cloacae*.

Keywords: Cashew Apple Bagasse, Hydrogen, Nitrogen source, *Enterobacter cloacae,* Dark fermentation

1 INTRODUCTION

Given the concern about climate change, carbon dioxide (CO_2) is the most common gas among greenhouse gases, directly impacting the planet's temperature[1]. One of the possibilities to mitigate CO_2 emissions is using hydrogen generated from technological processes that use renewable sources[2]. Therefore, hydrogen production using biomass as feedstock becomes quite interesting, mainly due to the possibility of the use of several sources, for example, lignocellulosic biomass, sludge organic industrial waste, food residues, and crop residues[3].

Biohydrogen is produced as a byproduct of the metabolic pathways of some microorganisms for photofermentation, dark fermentation, thermophilic fermentation[4], and biophotolysis of water by algae/cyanobacteria[5]. The dark fermentation (DF) method allows the obtention of hydrogen via the metabolism of anaerobic microorganisms in the absence of light and oxygen, through the degradation of organic residues[6]. The bioprocess generally has a high rate of H_2 production compared to other biological processes, meaning a greater mass of H_2 despite its theoretical conversion being lower compared to, for example, microorganisms that produce H_2 with light[7]. Also, the DF system requires less energy expenditure, with light and oxygen control in fermentation.

The cashew apple bagasse (CAB), a by-product of the nut and cashew apple juice industry (*Anacardium occidentale* L.), presents itself as an abundant and cheap source of lignocellulosic biomass in Brazil, and it was evaluated as feedstock in DF process by Silva et al.[8] Theses authors studied the influence of pretreatments of CAB to obtain the culture medium in the H_2 production. However, the yield of H_2 was low. Then, a possible way to improve the results of this culture medium is to add nitrogen sources.[9]

In this context, this work aims to produce hydrogen by *Enterobacter cloacae* via dark fermentation using enzymatic hydrolysate from cashew apple bagasse, evaluating its supplementation with a nitrogen source.

2 MATERIAL & METHODS

Pretreatment and enzymatic hydrolysis of the cashew apple bagasse to obtain the culture medium: The cashew apple bagasse was purchased from Agroindustrial Cashew Cooperative of Ceará-Brazil. It was washed three times and dried at 60 °C for 24 h in Tecnal TE-397/4 ovens (Tecnal, Piracicaba, SP, Brazil). The dried bagasse (CAB-D) was then crushed and standardized between 20-80 mesh, and stored at room temperature (25 °C). After, the CAB-D undergoes a mild acid treatment, using 0.6 mol/L of H_2SO_4 in an Erlenmeyer flask containing 20% (w/v) solid at 121 °C for 30 min. The solid fraction, called CAB-AC, is retained and washed three times. After, CAB-AC was subjected to an alkali treatment using 1 mol/L NaOH and a ratio of 20% (w/v) CAB-AC at 121 °C for 30 min. The pretreated solid, composed mainly of cellulose, was washed until pH 7, dried at 50 °C for 24 h, crushed, and standardized between 0.84–0.18 mm. Then, pretreated bagasse was enzymatically hydrolyzed at 45 °C and 150 rpm for 72 h using a solid load of 8% (w/v) and an enzyme load of 15 FPU/g cellulase from *Trichoderma reesei* (Sigma Aldrich, 77 FPU/mL) and 30 CBU/g cellulose NS-50010 β-glucosidase (Novozymes 188 CBU/mL), in sodium citrate buffer 50 mM at pH 4.8. After, the system was centrifuged at 7000 rpm for 20 min at 4 °C and the collected supernatant was filtered through 22 µM. The concentration of glucose from CAB-HE was determined by High-Performance Liquid Chromatography (HPLC). Then, the

CAB-EH was diluted to obtain a glucose concentration of 20 ± 4 g/L, and it was evaluated the supplementation with 3 g/L peptone and 3 g/L urea. The culture media were sterilized in autoclave at 110 °C for 10 min.

Microorganism and preparation of the inoculum: The bacteria used in this work was *Enterobacter Cloacae* ATCC 13047, acquired from the André Tosello foundation. The strain was activated using nutrient agar medium (composed of 3 g/L meat extract and 5 g/L peptone, pH = 6.8 ± 0.2) and it was cultivated at 37 °C for 24 h without agitation and under light restriction.

Bioprocesses to produce the biohydrogen: The fermentation processes were carried out in 250 mL flasks (Brand Schott Duran®) at 37 °C and 100 rpm with a reaction volume of 200 mL. The media were inoculated with 10% (v/v) inoculum, corresponding to 0.47 g/L cell concentration. After the inoculation, the medium was purged with 0.5 L/min N_2 gas for 5 minutes to promote an anaerobic environment. Liquid samples were collected at predetermined times. The samples were centrifuged at 6000g for 15 min and filtered through 22 µM membranes for HPLC analyses. Gas bags were attached to the flasks, collecting the gas to determine the volume, and the composition was analyzed by GC. All experiments and analysis were done in triplicate.

Liquid and gaseous phases analysis: The microbial growth was determined by optical density at 600 nm (OD600) and the cell concentration (g/L) was calculated using a calibration curve. Samples from processes were centrifuged at 6000g for 15 min and the supernatant was used to determine the sugars, organic acids, and alcohols concentrations by High-Performance Liquid Chromatography (HPLC) (Thermo Finnigan Surveyor HPLC System), using a refractive index detector and the Supelco 610-H column. The eluent was 0.1% (v/v) H_3PO_4 at a flow rate of 0.5 mL/min and 30 °C with an injection volume of 20 µL. Biogas composition was determined by GC analysis, using a Shimadzu GC-2010 equipped with a TCD (thermal conductivity detector) and a Carboxen 1010 PLOT capillary column (fused silica) measuring 30 m x 0.32 mm.

3 RESULTS & DISCUSSION

The hydrogen production by *E. cloacae* was evaluated using CAB-EH (containing 20 ± 4 g/L glucose), CAB-EH supplemented with peptone, and CAB-EH supplemented with urea. The Figures 1 A-C present the behaviour of cell growth, glucose consumption, and the production of acetic acid, lactic acid and ethanol during 72 h. After 72 h, *E. cloacae* consumed 70.8 ± 2.1 % (Fig. 1A) ,100 ± 0 % (Fig. 1B) and 37.5 ± 2 % (Fig. 1C) of the glucose, achieving the maximum cell concentration of 0.63 g/L, 0.71 g/L and 0.16 g/L, in the bioprocesses using CAB-EH, CAB-EH + peptone, and CAB-EH + urea, respectively.

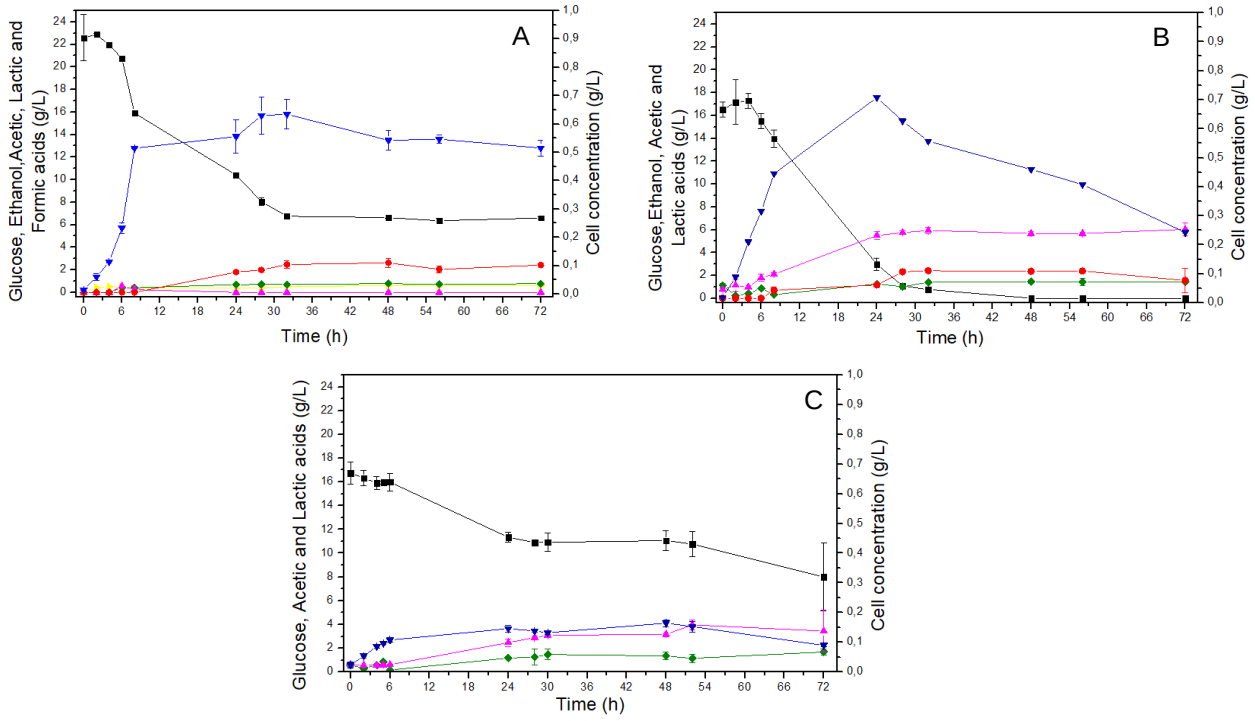

Figure 1. Concentration of *E. cloacae* cell (▼), glucose (■), acetic acid (▲), lactic acid (♦), formic acid (►) and ethanol (●) concentrations as function of time, obtained of the bioprocesses at 37 °C using (A) CAB-EH medium, and the CAB-EH medium supplementated with (B) peptone and (C) urea during 72 hours of fermentation.

Analysis of the liquid phases, evidenced that acetic acid, lactic acid, formic acid and ethanol were the main metabolite produced during the bioprocesses. The assay without supplementation (Fig 1-A) showed that the main metabolites were ethanol. Of the acids produced, lactic acid stands out as the main one produced in this assay, acetic acid appeared only withing 6 h of this bioprocess. However, in the assay using CAB-EH supplemented with nitrogen, the main metabolite was acetic acid and no formic acid was formed. The concentration of these organic acids in CAB-EH (Fig 1-A) had a maximum value of 0.55 ± 0.03 g/L for the acetic acid, 0.78 ± 0.02 g/L for the lactic acid, and 0.6 ± 0.03 g/L for the formic acid, and also was produced 2.6 ± 0.4 g/L of the ethanol. In the bioprocess using CAB-EH supplemented with peptone were produced 6.0 ± 0.5 g/L acetic acid, 1.4 ± 0.1 g/L lactic acid, and 2.4 ± 0.0 g/L ethanol, and supplementing the CAB-EH with urea was achieved 3.4 ± 1.8 g/L and 1.7 ± 0.2 g/L of acetic and lactic acids, respectively, and did not produce ethanol. The metabolic pathway of *E. cloacae was* studied by Sun et al.[10], and the authors reported that these are of metabolism of the *E. cloacae*.

In the Table 1 are showed the glucose consumption, productivity (P_{H2} (mL/$g_{glucose}$)), yield (Y_{H2} (mL/$g_{glucose}$)), molar yield (mol H_2/mol glucose) of the bioprocesses conducted at 37 C°, 100 rpm with 24 h.

Table 1 Summarized results from the assays in the first 24 hours of fermentation.

Assay	Glucose consumption (%)	Max biogas(mL)	Produced Gases (%)		Max volume of H_2 (mL)	Y_{H2} (mL/$g_{glucose}$)	P_{H2} (mL H_2/L.h)	Mol H_2/mol glucose
			H_2	CO_2				
CAB EH	53,7± 4.5	851 ± 18.4	29.9 ± 1.4	70.1 ± 1.4	236 ± 21.4	21.1	54.6	0,14
CAB EH + urea	32.6 ± 4.5	104 ± 4.7	14.2 ± 2.6	85.8 ± 2.6	8.5 ± 0.85	1.6	1.9	0.01
CAB EH + peptone	82 ± 2.5	1094 ± 39.6	25.6 ± 5.6	74.4 ± 5.6	257.8 ± 61.3	19.56	59.7	0.14

The highest production of biogas (1096.5 ± 38.9 mL) was observed using the CAB-EH supplemented with peptone (Table 1) and produced 1432.22 mL of H_2/ L of medium. However, the lowest production of biogas was obtained using the CAB-EH supplemented with urea, producing 109.0 ± 7.9 mL of biogas and produced 47,22 mL of H_2/ L of medium, much lower than that obtained in the process using the CAB-EH without supplementation (899.0 ± 32.5 mL, which produced 1331.11 mL of H_2/ L of medium). Peptone is obtained from protein hydrolysis, resulting in a complex product rich in amino acids, vitamins, inorganic salts, sugars, and lipids, however, the urea is a single compound. The highest biogas production occurred in the first 24 hours, which indicates that hydrogen production occurs in the exponential phase of *E. cloacae* growth. As peptone is a set of substances organic and inorganic, this contributes positively to the metabolism of *E. cloacae*, promoting the production of hydrogen, whereas with urea, it negatively interferes with the metabolism, corroborating with the lowest cell growth. This probably indicates that the carbon-nitrogen ratio was not ideal. The peptone used in this experiment had a composition ≥ 14% nitrogen, while in urea this composition was 46.6% nitrogen Then, the addition of equal masses of different nitrogen sources resulted in a ratio C/N different for both experiments, and in the case of urea it didn't work very well.

The H_2 production obtained using CAB-EH, CAB-EH + peptone, and CAB-EH + urea was 9,7± 0,9 mmol, 10.6 ± 2.5 mmol, and 0.4 ± 0.03 mmol, respectively. The cumulative hydrogen was 54 mmolH_2/L of hydrolysate using CAB-HE 20 g/L glucose, 58.9 mmolH_2/L of hydrolysate using CAB-EH + peptone and only 2 mmolH_2/L of hydrolysate using CAB-EH glucose + urea. These results were influenced by the production of organic acids[11]. It is known that the production of these acids as well as a decrease in pH in the environment impair the route to hydrogen production. The final pH was 4.7, 5.1, and 5.3 in the bioprocesses conducted with CAB-EH without supplementation, supplemented with urea and with peptone, respectively. Furthermore, for the theoretical production of hydrogen using glucose as a source to come as close to the theoretical limit, it is ideal that acetic acid should be the only product, however in the test without supplementation the main fermentation by-product was lactic acid. In trials with nitrogen supplementation, the added nitrogen favored the production of acetic acid as the main byproduct.

Another study has already evaluated the production of hydrogen using cashew apple bagasse as feedstock by Silva et al.[8] using hydrolysates from cashew apple hydrolysates obtained by different techniques, and the best result of hydrogen production by *Clostridium roseum* was achieved using the acid hydrolysate of cashew apple bagasse, producing 15 mmolH_2/L in 24 h. In the present study, the hydrogen production by *Enterobacter cloacae* was 58.9 mmolH_2/L of hydrolysate using CAB-EH supplemented with 3 g/L peptone, a higher production

4 CONCLUSION

This work investigated the effect of nitrogen sources to improve the hydrogen production by *Enterobacter cloacae* for dark fermentation using cashew apple bagasse hydrolysate as culture medium. The highest cumulative H_2 production (1096.5 mL/L) was obtained using peptone as a nitrogen source, indicating that nitrogen sources can improve H_2 production. This study is promising and must be continued to hydrogen that came from a biological route with a price competitive in opposition to methods that use fossil sources.

REFERENCES

1 Nemitallah A.M., Alnazha A.A., Ahmed U., El-Aldawy M., Habibib A.M., 2024. RINENG. Vol. 21. 101890
2 Sarmah M.K., Singh P.T., Kalita P., Dewan A., 2023. RSC Advances, Vol. 13, Issue 36, 25253-25275
3 Guellout Z.,Francois-Lopez E., Benguerba Y., Dumas C. ,Yadav K. K.., Fallatah M. A., Pugazhendhi A., Ernst B., 2022, J. of Environ. Manag., 114393
4 Saravanan A..,Kumar S. P., Khoo S.K.,Show P., Carolin F.C.,Jackulin F.C.,Jeevanantham S., Karishma S., Show K.,Lee D., Chang J. 2021. Bioresour. Technol. Vol. 342. 126021
5 Das D., Veziro glu N.T., 2001. Int. J. of Hydrogen Energy. Vol. 26. Issue 1. Pages 13-28
6 Lacroux J.,Llamas M., Dauptain K, Avila R., Steyer J., Lis R., Trably E.,2023. Sci. Total Environ. Vol.865. 161136
7 Chandran, E.M., Mohan, E., 2023. Eviron Sci Pollut Res. 30, 102129 -102157
8 Silva J.S., Mendes J.S., Correia J.A.C., Rocha M.V.P., Micoli L., 2018, J. of Biotechnol., Vol. 286, Pages 71-78
9 Hamilton C., Calusinska M.,Baptiste S., Masset J., Beckers l., Thonart P.,Hiligsmann S.,2018, Int. J. of Hydrogen Energy, Vol. 43. Issue 11. Pages 5451 -5462
10 Sun L., Huang A.,Gu W, Ma Y,Zhu D., Wang G., 2015, Int.l J. of Hydrogen Energy., Vol. 40. Issue 3, Pages 1402-1407
11 Yin Y.,Song W., Wang J,. 2022, Bioresour. Technol., Vol.364. 128074

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